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Paul Lueth

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POLYANYDRIDE NANOPARTICLES: A DRUG DELIVERY VEHICLE TO KILL INTRACELLULAR PATHOGENS

PAUL LUETH
DEPARTMENT OF VETERINARY MICROBIOLOGY AND PREVENTIVE MEDICINE
IN PARTIAL FULFILLMENT OF NON-THESIS MASTERS DEGREE
INTERDISCIPLINARY GRADUATE STUDIES PROGRAM
JULY 14, 2015
Overview

• Introduction to IGS
  • VMPM
  • IMBIO
  • CHEM E

• Project Outline

• Key Observations, Results, Conclusion

• Future Plans
Interdisciplinary Graduate Studies Program

DESCRIPTION
Available to graduate students who wish to have a more diversified program of advanced study than generally permitted for students who specialize in a single subject.

Program is open to any qualified graduate student, who wish to improve their subject matter competence in more than one discipline.

COURSEWORK
Allowed to take courses in three different graduate subject matter areas.

Each subject contributing a minimum of nine (9) semester credits toward the 35 semester graduate credits required for the degree.

NON-THESIS OPTION
A creative component is required in which the student demonstrates independent creativity such as a written report of laboratory, field or library research, a project in fine arts, or some other original contribution.
The student, in consultation with his/her Program of Study Committee, will decide on the choice of option (i.e., thesis or non-thesis). The Program of Study Committee also aids the student in planning a program of study, selecting appropriate courses, and determining foreign language requirements, if applicable.
Interdisciplinary Graduate Studies Program

CHEM E

VMPM

IMBIO
Interdisciplinary Graduate Studies Program

- KILLING
- PATHOGEN
- DISEASE
- THERAPY
- SAFETY
- HOST
Brucellae

Gram Negative
Coccobacillus
Faculative Intracellular Pathogens:
10 Species
<table>
<thead>
<tr>
<th>Species</th>
<th>Biovar/Seravar</th>
<th>Natural Host</th>
<th>Human Pathogen</th>
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</thead>
<tbody>
<tr>
<td>B. melitensis</td>
<td>species</td>
<td>Goat, sheep</td>
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<tr>
<td>B. abortus</td>
<td>1-6,9</td>
<td>Cattle, bison, buffalo</td>
<td>yes</td>
</tr>
<tr>
<td>B. suis</td>
<td>1, 2, 3</td>
<td>Swine</td>
<td>yes</td>
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<td></td>
<td>4</td>
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<td>Yes</td>
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<td>Reindeer, caribou</td>
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<td>Dogs, other canids</td>
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<td>B. ovis</td>
<td>none</td>
<td>Sheep</td>
<td>no</td>
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<td>B. neotomae</td>
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<td>Rodents</td>
<td>no</td>
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<td>B. maris</td>
<td></td>
<td>Marine mammals</td>
<td>Yes(?)</td>
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<td>B. pinnipediae</td>
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<td></td>
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</tr>
<tr>
<td>B. cetaceae(?)</td>
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</table>
Human Transmission

Rarely Fatal

Not transmissible from person to person

Zoonotic

Highly infection through aerosol

10-100 bacteria for infection

Conjuctiva, skin lesions, contact with infectious tissue, ingestion of unpasteurized dairy

http://www.vet.uga.edu/vpp/archives/ivm/ENG/zoonoses/domestic/images/brucella_img02.jpg
Brucellae

Symptoms in Humans
Undulating Fever
Headache
Anorexia
Complications include endocarditis, meningitis, arthritis
Brucella

Treatment

Daily Combination of Doxycycline & Rifampin

Regiment 6-8 weeks

Relapse

No Vaccine for Human Brucellosis
Prevention

• Animal Vaccines
• Avoid direct contact with visibly sick animals
• Wear eye protection and gloves when handling carcasses
• Do not drink unpasteurized milk
Brucellae

Biological Threat

CDC Category B Biological Threat Agent

- Debilitating Symptoms
- Significant Morbidity
- Relapse of Infection
- Lack of Human Vaccine
Virulence Factors

Intracellular nice
LPS very little endotoxicity & poor inducer of cytotoxic mediators
Smooth O-chain are resistant to serum & complement, enter via lipid rafts
Type IV Secretion System
Cyclic β-1,2-glucans
Prevents Apoptosis
(2,3-DHBA) & 2,3-DHBA-based siderophore brucebactin (Bellaire et al, 2003).
1. Macrophage moving toward bacteria.

2. Bacteria being engulfed.

3. Bacteria contained within a vesicle.

4. Lysosome fusing with a vesicle and releasing phagocytic enzymes.

5. Bacteria being destroyed and digested.

6. Undigested remains of bacteria.

EEA-1

pH=4.5

Vacuolar ATPase

pH=4.0

Cathepsin D

pH=6.5

Rab5

pH=6.5

Lamp-1

Rab7

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3. Bacteria contained within a vesicle.

4. Lysosome fusing with a vesicle and releasing phagocytic enzymes.

5. Bacteria being destroyed and digested.

6. Undigested remains of bacteria.

1. Macrophage moving toward bacteria.

2. Bacteria being engulfed.

Golgi apparatus
nucleolus
nucleus
mitochondrion
ribosomes
endoplasmic reticulum
lysosome

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Brucella Host Cellular Immune Response

Effective Response requires CMI

Cell-Mediated Response
- $T_H1$ response
- Activate Macrophages

Immunity is based on IFN-γ
- Controlled by IL-12 and TNF-α

ROI & NO contribute to Control
Drug Delivery

“refers to approaches, formulations, technologies, and systems for transporting a pharmaceutical compound in the body as needed to safely achieve its desired therapeutic effect.”

Therapeutic Index

- Maximum Desired Level
- Minimum Effective Level

Drug Levels in Circulation

- Dose 1
- Dose 2
- Dose 3
- Dose 4
- Dose 5
- Dose 6

Time
Detriments of Soluble Therapy

Drug Levels in Circulation

Maximum Desired Level

Minimum Effective Level

Time

Dose 1
Dose 2
Dose 3
Dose 4
Dose 5
Dose 6
Controlled Drug Delivery

Drug Levels in Circulation

Time

Maximum Desired Level

Minimum Effective Level

Dose 1
Drug Delivery
Must stabilize drug

Drug must be bioactive.
Therapeutic drugs (proteins), prone to deactivation (synthesis)
Proteins, in soluble form, may lose their functional properties (in vivo)

Administer drug at a concentration
necessitates therapy
precludes toxicity

Drug to be released in a controlled, and sustained manner.

Material that encapsulates and delivers drug should be biodegradable
Biodegradable Polymers

- Encapsulates drug
- Safely degrade
- Excellent candidates for controlled release
- Versatility in degradation mechanism
- Two main classes: Polyanhydrides and Polyesters
- Two degradation mechanism
What can be hydrolyzed?

1. Anhydrides

2. Esters

3. Carbonates

4. Amide
Polyesters vs. Polyanhydrides

Both approved for use in humans
Both have tailorable release rates
Polyesters: bulk eroding, acidic degradation products
Polyanhydrides: surface eroding, very hydrophobic
Polyanhydrides

- Anhydride bond
  - Hydrolytically unstable
  - Degrades into two carboxylic acid groups
- Suitable for drug delivery systems
  - Gliadel®
- Surface erodible

# Commercialized Controlled Release Formulations

<table>
<thead>
<tr>
<th>Brand Name</th>
<th>Application</th>
<th>Release time</th>
<th>Delivery route</th>
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</thead>
<tbody>
<tr>
<td><strong>GLIADEL WAFER</strong> (polifeprosan 20 with carmustine implant)**</td>
<td>Anti-tumor</td>
<td>Weeks</td>
<td>Implant</td>
</tr>
<tr>
<td><strong>Claritin-D 24Hour</strong></td>
<td>Allergy</td>
<td>1 Day</td>
<td>Oral</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td>Contraceptive</td>
<td>1 Month</td>
<td>Injection</td>
</tr>
<tr>
<td><strong>PROZAC Weekly</strong></td>
<td>Anti-depressant</td>
<td>1 Week</td>
<td>Oral</td>
</tr>
</tbody>
</table>
Bulk Erosion

- Hydrophilic backbone
- Porous
- Allow water to diffuse through surface
- High porosity
- Fast process

Modified 1,8-bis(\(p\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG)
Modified

1,8-bis(\(p\)-carboxyphenoxy)-3,6-dioxaoctane (CPTEG)
Surface Erosion

Surface Erosion
Hydrophobic polymers
Prevent water from penetrating interior
Limited porosity
Slow process

Hydrophobic anhydride monomer
1,6-bis(\(\rho\)-carboxyphenoxy) hexane (CPH)
Hydrophobic anhydride monomer
1,6-bis(\(\rho\)-carboxyphenoxy) hexane (CPH)
Erosion Mechanism of CPTEG:CPH

Polymers exhibit distinct erosion profiles that can be controlled by tailoring copolymer composition

Chemistry Matters...

Polymer degradation
- Bulk erosion (e.g., CPTEG)
- Surface erosion (CPH & SA)

Drug/protein release

Protein/antigen stabilization
- Structure
- Function

Immunomodulatory vaccines
- Single dose
- Efficacious immune responses

20:80 copolymer  50:50 copolymer

Wilson Weldter et al 2006
Project Goal

Develop and assess polyanhydride nanoparticles as an intracellular antibiotic delivery vehicle.
1. Macrophage moving toward bacteria.
2. Bacteria being engulfed.
3. Bacteria contained within a vesicle.
4. Lysosome fusing with a vesicle and releasing phagocytic enzymes.
5. Bacteria being destroyed and digested.
6. Undigested remains of bacteria.

Golgi apparatus
nucleolus
nucleus
mitochondrion
ribosomes
endoplasmic reticulum
lysosome

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Time course of *B. melitensis* in Raw Cells

![Graph showing the time course of *B. melitensis* in Raw Cells. The graph includes bars for different time points (T=0, T=24, T=48, T=72) and a trend line indicating the change over time. The CFU/ML values are plotted on a logarithmic scale.](image)
Intracellular Efficacy of PA nanospheres encapsulated w/22% Doxycycline, against *B. melitensis* in RAWS, 48 Hrs Post-treatment

* p < 0.05c
Results

• Encapsulated PA nanoparticles were effective
  • Depot
  • Controlled release
  • Reduced/eliminated bacterial load

• 20:80 CPH:SA more effective after 24HRS

• 20:80 CPTEG:CPH more effective after 48 HRS, with no viable CFU
Key Observation

• At later time points, the number of cells containing nanoparticles decreased with infected cells.
• Within the phagosome, there was limited colocalization between nanoparticles and *Brucella*.
• Nanoparticles and/or *Brucella* were not always localized within the phagosome.
Incorporate encapsulated & blank nanoparticles in In vitro *Brucella* killing assay.
Hypothesis
1. Colocalization between *Brucella* and NPs (antibiotics + dye), would not occur, as the NP arrive at their depot and releasing antibiotic, which kills *Brucella*, overtime and eliminating any GFP fluorescence.

2. Colocalization between *Brucella* and NP (dye alone), would occur, as the NPs arrive at their depot but do not contain antibiotic. Allowing *Brucella* to survive and fluoresce GFP.
**Brucella melitensis** in RAW 264.7 CELLS, 72 HRS P.I.

Colocalization of *B. melitensis* (*GFP*) and LAMP-1 (*Cy5*)

<table>
<thead>
<tr>
<th>ROI</th>
<th>Area (pixels²)</th>
<th>Pearson’s Coeff.</th>
<th>Overlap</th>
<th>Overlap Index 1</th>
<th>Overlap Index 2</th>
<th>Coloc. Index 1</th>
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<tbody>
<tr>
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<td>0.43717</td>
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**Brucella melitensis** in RAW 264.7 CELLS, 2 HRS P.T. (CC)
Colocalization of *B. melitensis* (GFP) and LAMP-1 (Cy5)

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<td>0.0025612</td>
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**Brucella melitensis** in RAW 264.7 CELLS, 24 HR P.T. (CC)

Colocalization of *B. melitensis* (GFP) and Nanoparticles with Antibiotic *(Rhod-B)*

<table>
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**Brucella melitensis** in RAW 264.7 CELLS, 24 HR P.T. (CCB)

Colocalization of *B. melitensis (GFP)* and Nanoparticles without Antibiotic (*Rhod-B*)

<table>
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**Brucella melitensis** in RAW 264.7 CELLS, 24 HR P.T. (CS)
Colocalization of *B. melitensis* (*GFP*) and Nanoparticles with Antibiotic (*Rhod-B*)

<table>
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<th>Coloc. Index 1</th>
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Brucella melitensis in RAW 264.7 CELLS, 24 HR P.T. (CSB)

Colocalization of B. melitensis (GFP) and Nanoparticles without Antibiotic (Rhod-B)

<table>
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<th>ROI</th>
<th>Area (pixels^2)</th>
<th>Pearson's Coeff.</th>
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Results

• At 24 hours post treatment blank nanoparticles (CCB/CSB) had significantly larger Pearson Coefficients compared to (CC/CS)

• 20:80 CCB Pearson Coefficient Average (0.713)
• 20:80 CSB Pearson Coefficient Average (0.927)
Key Observation

• Although nanoparticles were observed in phagosome, many were outside of cells, sometimes causing a lot of background in microscopy images.
Key Observation

• With microscopy, there was difficulty quantifying monolayer sub-population (High-Low-No Infection)
• Difficulty gating populations
UTILIZING MULTI-SPECTRAL IMAGING FLOW CYTOMETRY TO QUANTIFY THE INTERNALIZATION/COLOCALIZATION OF POLYANHYDRIDE NANOPARTICLES OR BACTERIA IN RAW 264.7 CELLS
Brucella melitensis infection in RAWS

- High Infection: 18.43, 16.9, 35.91, 60.67
- Low Infection: 53.12, 32.74, 30.65, 15.73
- Non-Infected: 28.45, 50.36, 33.44, 23.6
Graphs showing high and low intensity of GFP over time:

- **T = 0**: Initial state with high and low intensity distributions.
- **T = 26**: Intensity levels remain similar to initial state.
- **T = 48**: Further observation shows slight changes in intensity distribution.
- **T = 72**: Final state with minimal changes from previous observation.

Legend: GFP High Intensity, GFP Low Intensity.
ImageStream Significance

IS capable of detecting both internalized nanoparticles as well as bacteria, and excluding external components.

IS capable of gating and generating robust statistical data about subpopulations within an infection profile.

IS capability provides an accurate cell-by-cell analysis of fluorescent signal intensities and spatial relationships (colocalization) between different structures and cellular features at high speed.
Conclusions

• Encapsulated PA nanoparticles are effective therapeutic agents capable of reducing/eliminating bacterial load in murine macrophages.
  • PA nanoparticles localize in phagosomes.
  • PA nanoparticles safely deliver antigen.
  • PA nanoparticles can control release of antigen.
  • PA nanoparticles chemistry modifications alter therapeutic profile.
Future Directions: *Burkholderia* spp.

*B. pseudomallei* $T = 24$ PT with NP doxycycline

**CFU/ML**

<table>
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<tr>
<th></th>
<th>UT</th>
<th>SOL</th>
<th>CC</th>
<th>CS</th>
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</tbody>
</table>

* $p < 0.05c$
Acknowledgements

• God, Family (Wife-Patience), Kids, (Lucas, Manny, Amara, Moses-Michae, Yaa’El)
• Parents (Mama/Baba), siblings (Bona, Rebecca, Helen, Moses, Mike, Mat, Ben)
• Church (HVC, Josh Miller, Phil Penner, Lawsons)
• Babysitters (Brianna Smith, Yvonne & Nicole Kemei, Apal Wol, Mwape, Erin Gillian)
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